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The Connexin Turnover, an Important Modulating Factor of the Level of Cell-to-Cell Junctional Communication: Comparison with Other Integral Membrane Proteins

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Abstract The constituent proteins of gap junctions, called "connexins" (Cxs) in chordates, are generally renewed several times a day, in approximately the same rate range as many other integral plasma membrane proteins and the proteins of other channels, other intercellular junctions or different membrane receptors. This permanent renewal turns on a fine-tuned balance among various processes, such as gene transcription, mRNA stability and processing, protein synthesis and oligomerization, post-translational modifications, transport to the plasma membrane, anchoring to the cytoskeleton, connexon aggregation and docking, regulation of endocytosis and controlled degradations of the proteins. Subtle changes at one or some of these steps would represent an exquisite level of regulation that extends beyond the rapid channel opening and closure events associated with channel gating; membrane channels and receptors are constantly able to answer to physiological requirements to either up- or downregulate their activity. The Cx turnover rate thereby appears to be a key component in the regulation of any protein, particularly of gap junctional proteins. However, the physiological stimuli that control the assembly of Cxs into gap junctions and their degradation remain poorly understood.

Keywords Gap junction · Connexin · Connexin half-life · Intracellular trafficking

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Introduction

Cells strictly regulate expression of a wide variety of integral proteins present in their surface membranes to achieve precise yet dynamic control of their functions. The extent to which cells of different tissues are coupled is determined by multiple mechanisms, including tissue-specific patterns of expression of different connexins (Cxs) and regulatory pathways that control their synthesis, intracellular trafficking, assembly into intercellular channels and degradation.

The activity of gap junction channels (as the one of the other channels present in cell membranes), expressed by their macroscopic conductance (G), is determined by three factors, the number of present channels, their open probability (P_o, the fraction of time spent in the open state) and their unitary conductance (γ) activity. Each of them is independently modulated by multiple factors, which may have apparently opposite effects. Channels made of Cx43, e.g., were seen to undergo increases of their unitary conductance (Takens-Kwak & Jongsma, 1992; Moreno et al., 1994) and decreases of their macroscopic conductance (Verrecchia et al., 1999) when cells were exposed to dephosphorylating treatments. Single-channel events are frequently observed after treatment of cells with chemical agents (e.g., heptanol, etc.) considered to reduce the time spent by the channel in the open state without modifying the other characteristics of the channel (Burt & Spray,

1988). In contrast with the other channels present in the membranes, the open probability of gap junction channels cannot be experimentally determined. The existence of a permanent cell-to-cell communication was for a long time regarded as a sign of a relatively high open probability, but Bukauskas et al. (2000) observed that in transfected cell pairs, even in plaques large enough to mediate coupling, only a small fraction of channels were simultaneously open – in other words, they exhibited a low open probability.

Several membrane channels, including gap junction channels, have been seen to exhibit relatively rapid turnover kinetics; and this mechanism might be important in the regulation of their activity. Each cell protein has a defined intracellular stability. Its amount is the outcome of the opposing processes of synthesis (i.e., transcriptome) and degradation (i.e., metabolome). The regulation of protein expression indeed depends on a fine-tuned balance among various processes, such as gene transcription, mRNA processing, protein synthesis and assembly, post-translational modifications, transport to the cell surface, anchoring to the cytoskeleton, regulation of endocytosis and controlled degradation of the protein. Subtle changes at one or some of these steps would represent an exquisite level of regulation that extends beyond the rapid channel opening and closure events associated with channel gating.

The present review summarizes the available data concerning the turnover kinetics of Cxs, compares them with the data of some channels and transmembrane proteins present in the plasma membrane and discusses their functional importance. The half-life of proteins ($t_{1/2}$, i.e., the time required for their decay of one-half in a biological system) being much shorter than the half-life of the cells, the rate of protein turnover is critical for the steady-state level of surface expression and is tightly coupled to signaling events.

Methodological Approaches

A classical approach for estimating protein renewal rates involves exposure of a cell or organism to labeled precursors, followed by immunoprecipitation of proteins of interest. A difficulty is the possible reutilization of the amino acids, thus giving longer apparent half-lives (*see*, e.g., Yancey et al., 1981). ^{14}C-Bicarbonate, ^{35}S-methionine and ^{3}H-leucine are the most commonly used of the protein precursors.

A wide variety of intracellular localization and trafficking issues can now be addressed in living cells by the development of green fluorescent protein (GFP) as an effective chromophore, producing a strong green fluorescence that does not require other gene products, is not limited by availability of substrates and does not apparently interfere with cell growth and function. Cx polypeptides were tagged on their C termini with GFP or its cyan (CFP) and/or yellow (YFP) color variants, and the tagged Cx trafficking and assembly into functional gap junction plaques were investigated by single-, dual-, or triple-color deconvolution microscopy. The principal limitations of this technique might concern protein interactions. For instance, the presence of GFP might mask interacting domains of the Cx tail extremity such as those interacting with ZO-1, which may be important for the stability of the gap junction plaques.

However, these methodological approaches are not applicable to all preparations. Some tissues, such as mature lens fibers in the developing lens nucleus, have lost their nuclei and protein synthetic machinery, are refractory to metabolic labeling and are therefore not represented in the present comparisons. The relatively slow turnover of different membrane receptors is often investigated by means of their irreversible chemical inactivation (e.g., by an alkylating agent) followed by quantification of binding site recovery parameters.

Cxs Are Generally Renewed Several Times a Day

One of the first attempts to estimate the renewal rate of gap junction proteins was carried out in the liver of rats having received a single injection of radiolabeled amino acid precursors. Gap junction proteins exhibited an apparent half-life of about 19 h (Yancey et al., 1981), but these authors suggested that this apparent $t_{1/2}$ probably overestimated the true half-life because of probable reutilizations of radiolabeled amino acids. The metabolic turnover of a given protein in cell culture or in organ culture does not necessarily reflect the dynamic state of this protein present in the organ, as emphasized by Jiang & Goodenough (1998). In lens organ cultures, which more closely mimic the *in vivo* process, 90 h are necessary for complete degradation of Cx45.6 and Cx56 *vs.* 16 h in lens primary cultures (Jiang & Goodenough, 1998). The latter authors hypothesized that a connexon pool refractory to turnover could not be labeled during the time course of the experiment. In mouse lens, another membrane channel, aquaporin-0 (AQP0, also known as major intrinsic protein, MIP), specifically present in fiber cell membranes, was also reported to be remarkably stable over a 24-h period (Sidjanin et al., 2001).

Cx half-lives reported in the literature, summarized in Table 1, range, except in one case, from 1.3 to 10 h. In one case (Cx56 present in cultured chicken embryo lens), the existence of two different kinetic pools, with half-lives of ≤2–3 h and ≤2–3 days, was reported, both the pools belonging to the same cellular localization (the plasma

Table 1 Comparison of the half-lives reported for different Cxs (or modified Cxs)

Cx	Modified Cx	Half-lives (h)	Cells or tissues[a]	Reference
Cx26		5	Adult mouse hepatocytes	Fallon & Goodenough, 1981
		1.3–2	Cultured mouse hepatocytes	Traub et al., 1989
Cx31		4.1	HeLa cells	Diestel et al., 2004
		6	*HeLa cells*	He et al., 2005
Cx32		4–6	Rat hepatocytes	Traub et al., 1983
		2.5–3	Mouse embryo hepatocytes	Traub et al., 1987
		~3	PC12 cells	VanSlyke et al., 2000
	Cx32T-GFP	3.3	*Hepatocellular carcinoma-derived PLC cells*	Windoffer et al., 2000
	Flagged Cx37	3	*BWEM cells*	Larson et al., 2000
Cx43		1.5–2	*Mouse sarcoma 180 cells and fibroblasts L929*	
		1.5	Chick lens epithelial cells in culture	Musil et al., 1990
		2–2.5	Normal rat kidney (NRK) cells	
		3.1	Novikoff hepatoma cells	Lampe, 1994
		~1.5	BICR-M1Rk cells	Laird et al., 1995
		2.5	E36 Chinese hamster ovary cells	Laing & Beyer, 1995
		3.27	Bovine aortic endothelial cells in culture	Larson et al., 1997
		2.7	Cultured leptomeningeal cells of newborn rat	Hertzberg et al., 2000
		2.66 ± 0.66 to 3.95 ± 0.88	MC3T3 osteoblastic cells	Yamaguchi & Ma, 2003
		2	SK-HEP-1 cells	Thomas et al., 2003
		~1	*BWEM cells*	Laing et al., 1997
		2.3	Bovine retinal endothelial cells	Fernandes et al., 2004
		1–2	Newborn rat cardiac myocytes	Laird et al., 1991
		1.9	Newborn rat cardiac myocytes	Darrow et al., 1995
		1.4	Newborn rat cardiac myocytes	Laing et al., 1998
		1.3	Adult rat heart	Beardslee et al., 1998
	Cx43-GFP	2–3	*HeLa cells*	Hunter et al., 2005
Cx45		4.2	*HeLa cells*	Hertlein et al., 1998
		2.9	Newborn rat cardiac myocytes	Darrow et al., 1995
Cx45.6		2.5	Lens of embryonic chicken in culture	Yin et al., 2000
	Cx49-GFP	10	*HeLa cells*	Breidert et al., 2005
Cx56		2–3 (1st pool)	Chicken lens cultured cells	Berthoud et al., 1999
		48 (2nd pool)		

Cx32T-GFP is a carboxy-terminally truncated version of rat Cx32 and enhanced-GFP chimera

[a] Roman characters, cells where Cx was endogenously expressed; italics, cells where Cx was exogenously expressed, surexpressed or mutated

membrane) and both corresponding to gap junction plaque-forming Cx56 (Berthoud et al., 1999).

Hunter et al. (2005) observed that both native Cx43 and Cx43-GFP expressed in HeLa cells turned over at similar rates (with half-lives of 2–3 h), showing that the C-terminal tagging of Cx43 does not interfere with Cx turnover in this cell model; it was similarly observed that the GFP tag did not affect cystic fibrosis transmembrane conductance regulator (CFTR) turnover (*see* Zhang et al., 2003).

Data concerning the turnover of the other gap junction proteins (innexins and pannexins) are still very scarce; Curtin et al. (2002) reported preliminary experiments that show that innexins have a half-life of ~6 h in the *Drosophila* optic lamina.

Proteins of Other Membrane Channels Also Exhibit a Relatively Large Range of Turnover Rates

Gap junction channels are one among a wide variety of transmembrane channels present in plasma membranes, macromolecular protein complexes embedded in the lipid bilayer and containing aqueous central pores allowing the passive passage of ions and sometimes of small molecules. The half-lives reported for some voltage-gated channels or ligand-gated channels, presented in Table 2, ranged from 40 or 50 min to 34 h or even, in one case, 14 days, for nicotinic acetylcholine receptors at neuromuscular junctions in living adult mice (Akaaboune et al., 1999). In the last study, the channel turnover was

Table 2 Examples of half-lives of channel-forming proteins of different channels present in the membranes, determined by pulse-chase approaches

Channel	Subunit	Half-lives	Cells or tissues[a]	References
Na^+ channels	Na	17 h	Rat pituitary GH3 cells	Monjaraz et al., 2000
	ENaC	1 h	MDCK cells	Staub et al., 1997
		40–50 min	A6 (*Xenopus* kidney epithelial) cells	May et al., 1997
		24–30 h	A6 cells	Kleyman et al., 2001
		1.04 ± 0.19 h	MDCK cells	Hanwell et al., 2002
K^+ channels	Kv1.3	3.8 ± 1.4 h	*Human embryonic kidney (HEK 293) cells*	Colley et al., 2007
	Kv1.4	87 min	*HEK 293 cells*	Jugloff et al., 2000
	Kv1.5	6.7 h	COS cells	Kato et al., 2005
	Kir1.1a (ROMK1)	<1 h (37°C)	*MDCK cells*	Brejon et al., 1999
		>4 h (26°C)		
Cl^- channels		>24 h	*K1 (Chinese hamster ovary, CHO) cells*	Lukacs et al., 1993
		24.1 h	*Apical membranes of MDCK cells*	Swiatecka-Urban et al., 2002
		12.9 h	*Basal membranes of MDCK cells*	
	CFTR	10.3 ± 2.3 h	*COS-7 (monkey African green kidney) cells*	Peter et al., 2002
		12 h	*NIH 3T3 (mouse fibroblasts)*	Zhang et al., 2003
		3 h	*Apical membranes of CFBE41o- cells*	Swiatecka-Urban et al., 2002
Aquaporin channels	AQP2	4 h	*CHO cells*	Tamarappoo & Verkman, 1998
	AQP4	24 h	*HEK cells*	Neely et al., 2001
NMDA receptors	CBP	14 ± 2.4 h	Synaptic membranes of rat brain	Chen et al., 1997
	GBP	18 ± 1.2 h		
	NR2A/B	16 ± 5 h		
	NR1	2 h (1st pool)	Rat cerebellar granule cells in culture	Huh & Wenthold, 1999
		34 h (2nd pool)		
	NR1	1 h (1st pool)	HEK 293 cells	Garcia-Gallo et al., 2001
		> 24 h (2nd pool)		
AMPA receptors	GluR2/3	18 ± 5 h	Rat cerebellar granule cells in culture	Huh & Wenthold, 1999
	GluR4	23 ± 8 h		
Nicotinic Ach receptors	nAChR	17 h	Cultured chick skeletal muscle	Gardner & Fambrough, 1979
	nAChR	14 days	Sternomastoid muscle of adult mice	Akaaboune et al., 1999

NMDA receptors, *N*-methyl-D-aspartate receptors, oligomeric complexes formed by the coassembly of members of three receptor subunit families: NR1 (a ubiquitous and necessary component of functional NMDA receptor channels), NR2 and NR3. The complex glutamate-binding protein (GBP) carboxypiperazine-4-yl-propyl-1-phosphonic acid (CPP)-binding protein (CBP) functions as an NMDA receptor in synaptic membranes. AMPA receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors, composed of four homologous subunits (GluR1, -2, -3 and -4), which assemble into a receptor complex, probably containing five subunits. CFTR, cystic fibrosis transmembrane conductance regulator channels; mAChR, muscarinic acetylcholine receptors; nAChR, nicotinic acetylcholine receptors; AQP2/AQP4, aquaporin-2/-4 channels

[a] Roman characters, cells where channels were endogenously expressed; italics, cells where channel proteins were exogenously expressed, surexpressed or mutated

considerably accelerated when neurotransmission was blocked (*see below*). In a few cases, the stability of some channels (e.g., Shaker potassium proteins expressed in HEK293T, which showed little degradation after 48 h; Khanna et al., 2001) precluded the determination of a $t_{1/2}$ value.

The existence of two different kinetic pools was reported for the NR1 subunit of *N*-methyl-D-aspartate (NMDA) receptors (Huh & Wenthold, 1999; Garcia-Gallo et al., 2001).

Comparison with Other Types of Integral Plasma Membrane Proteins

The protein turnover rate is a key component in the regulation of any protein since it allows response to alterations in physiological demands. The turnover kinetics varies between proteins. Hare & Taylor (1991) analyzed the degradation rate of 16 unidentified plasma membrane proteins of H4-II-E-C3 hepatoma cells and of 18 mouse 3T3 fibroblasts and noticed that, except in a few cases, they

frequently turned over slowly ($t_{1/2}$ > 75 h). A relatively wide range of half-lives has been reported for transmembrane proteins of different intercellular junctions, of different classes of membrane receptors or of membrane transporters, as presented in Table 3. Cxs (and probably innexins) – the main constituents of gap junctions and claudins and occludins, the principal tight junctional constituents – share similar topologies, with four α-helical transmembrane segments; all exhibit well-conserved extracytoplasmic cysteine residues that either are known to form or potentially can form disulfide bridges. Connexins, claudins and occludins appear to have half-lives in similar ranges, whereas a few membrane receptors or transporters turn over more slowly.

Factors Influencing the Measured Half-Lives

Identical plasma membrane proteins may have strikingly different turnover rates in different cell types; e.g., the low-density lipoprotein receptor is degraded with a $t_{1/2}$ of 15–20 h in fibroblasts but of 2–3 h in macrophages and the mannose-6-phosphate receptor is degraded with a $t_{1/2}$ of 16 h in Chinese hamster ovary cells but of 39 h in human leukemia cells (*see* Hare & Taylor, 1991, for discussion). In rat brain, the turnover rates of α_2-adrenoceptors showed marked regional differences, with half-lives of 2.1 days in the hypothalamus and corpus striatum and 2.6 days in the brainstem but 3.9 days in the cerebral cortex and 4.3 days in the hippocampus (Barturen & Garcia-Sevilla, 1992); and variations might still be more important in heterologous systems of expression. Weisz et al. (2000), e.g., observed that the half-life of the epithelial sodium channel (ENaC) that reached the plasma membrane of A6 cells was considerably longer than that of newly synthesized subunits; when these channels are expressed in *Xenopus* oocytes, the majority of them rapidly degraded shortly after synthesis and never reached the cell surface (<1% of the total ENaC was present at the plasma membrane at steady state; Valentijn et al., 1998). This observation reflects the fact that channel assembly may be differentially regulated in this overexpression system compared with its assembly in tissues that endogenously express the channel.

Studies on Cx turnover are frequently carried out in primary cultures of cells. After cell isolation, disaggregated cells contain intact gap junctions on their surfaces, coming from junctional plaques established with former neighbors. These gap junctions become internalized and disappear rapidly, whereas new gap junctions are formed. In these conditions, Cx synthesis and degradation rates might be increased compared with those in intact tissue. However, pulse-chase studies have shown that radioactive Cx43 disappeared in a monoexponential fashion with a similar half-life in, e.g., metabolically labeled rat hearts (Beardslee et al., 1998) and cultured myocytes (Laird et al., 1991; Darrow et al., 1995; Laing et al., 1998).

The situation appears particular in the lens, an avascular organ in which gap junctions play a pivotal role for cell physiology and transparency; superficial cells are more active in protein synthesis and degradation than the deeper fibers, where Cxs are refractory to metabolic labeling (*see* Jiang & Goodenough, 1998).

Disturbances in cellular metabolism frequently alter the Cx life span. As gap junction intercellular communication (GJIC) and Cx43 protein are decreased as a result of cytosolic acidification, Yamaguchi & Ma (2003) examined the influence of intracellular pH on the turnover of Cx43. The half-lives of the protein were 2.66 ± 0.66, 2.76 ± 2.17 and 3.95 ± 0.88 h at pH 6.9, 7.2, and 7.6, respectively; but the differences were not statistically significant. Hyperglycemia enhanced degradation of Cx43 in bovine retinal endothelial cells, reducing the half-life of the protein from 2.3 to 1.9 h, thus contributing to a decrease in GJIC (Fernandes et al., 2004). The authors suggested that Cx43 hyperphosphorylation in hyperglycemia would constitute the triggering signal for Cx43 degradation by a proteasome-dependent mechanism.

In a mammalian epithelial expression system (Madin-Darby canine kidney [MDCK] cells) maintained at a physiological temperature, wild-type ROMK1 is biosynthetically labile (half-time <1 h) and incapable of efficient traffic to the plasmalemma, suggesting that wild-type ROMK1 may require other factors, like the association of a surrogate subunit, for appropriate biosynthetic processing (Brejon et al., 1999). In contrast, reduced temperature had no effect on the half-life of wild-type CFTR (3.3 *vs.* 3.0 h in apical membrane of CFBE41o- cells; Swiatecka-Urban et al., 2005).

The variations in temperature and their incidence on the turnover rate of cell proteins, including gap junction proteins, are probably limited in homeotherms; but the situation is probably different in poikilotherms. Ducret et al. (2006) recently suggested that the innexin half-life might be significantly increased in the cold water-living lobster, and it seems reasonable to assume an opposite effect in animals living in a hot environment.

Protein phosphorylation is a key reversible posttranslational modification exploited by nature in profound ways to control various aspects of cell proliferation, differentiation, metabolism, survival, motility and gene transcription as well as the regulation of enzymatic activity, subcellular localization, complex formation and degradation of proteins. The increased phosphorylation of Cx43 induced by short-term 12-O-tetradeconylphorbol-13-acetate (TPA) treatment (<30 min) of Novikoff hepatoma cells, e.g., was accompanied by the reduced half-life of this protein from

Table 3 Examples of half-lives of transmembrane proteins, determined by pulse-chase approaches

Protein	Half-life	Cells or tissues[a]	References
Tight junction proteins			
Claudin-2	>12 h	MDCK cells	Van Itallie et al., 2004
Claudin-4	4 h		
Claudin-14	6 h	MDCK cells	Van Itallie et al., 2005
Occludin	11.2 h	MDCK cells	Chen et al., 2000
	~1.3 h	LLC-PK1 cells	Traweger et al., 2002
	2 h	IEC-6 cells	Guo et al., 2005
Adherens junction proteins			
E-cadherin	8 h	MDCK cells	Tsukamoto & Nigam, 1999
	6 h	SW48 colon carcinoma cells	Ireton et al., 2002
	15 h	IEC-6 cells	Guo et al., 2003
	7.9 h	HT29/B6 cells	Meyer zum Buschenfelde et al., 2004
Desmosome proteins			
Desmoglein 3	24 h	Human keratinocytes	Cirillo et al., 2006
Receptors			
Normal insulin receptors	9.9 h	Rat fibroblasts	Grako et al., 1992
EGF receptors	12 h	Monkey CV-1 cells	Kurten et al., 1996
	27 h	Rat mammary explants	Bolander, 1998
NGF receptors (Trka)	2.3 ± 0.06 h	PC12 cells	Jullien et al., 2002
VEGFR-2 receptors	70 min	Porcine aortic endothelial cells	Calera et al., 2004
Erythropoietin receptors	70 min	Ba/F3 cells	Yoshimura et al., 1990
	3 h	*HC-D57 cells*	Sawyer & Hankins, 1993
IL-6 (gp80, gp130) receptors	2–3 h	*MDCK cells*	Gerhartz et al., 1994
Vasopressin V2 receptors	3.6 ± 0.3 h	*COS-7 cells*	Martin et al., 2003
	11.52 ± 2.8 h	*MDCK cells*	Robben et al., 2004
Transferrin receptors	19 ± 6 h	HeLa cells	Rutledge et al., 1991
Human folate receptors	≥24 h	*Cell surface of CHO cells*	Chung et al., 1995
Muscarinic Ach receptors	27 h	Cultured heart cells	Waisberg & Shainberg, 1992
5-HT_{1A} serotonin receptors	109.2 h	Rat cerebral cortex	Vicentic et al., 2000
5-HT_{2A} serotonin receptors	113.2 h		
α_2-adrenoreceptors	2.1 days	Rat hypothalamus	Barturen et al., 1992
β_1-adrenoreceptors	9.4 h	C6 glioma cells	Neve & Molinoff, 1986
β_2-adrenoreceptors	6.4 h		
β_2-adrenoreceptors	12.6 h	L6 myoblasts	
D1 dopamine receptors	53 h	Rat cerebral cortex	Dewar et al., 1997
RPTP μ	3–4 h	Mink lung epithelial and T3 cells	Gebbink et al., 1995
Atrial natriuretic peptide receptors	7–8 h	Rat aorta smooth muscle cells	Hirata et al., 1986
ATP-dependent transport proteins			
PM Ca-ATPase	12 ± 1 days	Adult rat brain	Ferrington et al., 1997
α_1-subunit of Na,K-ATPase	35 h	MDCK cells	Rajasekaran et al., 2004

EGF, epidermal growth factor; NGF, nerve growth factor; VEGFR, vascular endothelial growth factor receptor; IL-6, interleukin-6; Ach, acetylcholine; PM Ca-ATPase, plasma membrane Ca-ATPase; RPTP μ, receptor protein tyrosine phosphatase μ; MDCK, Madin-Darby canine kidney; CHO, Chinese hamster ovary

[a] Roman characters, cells where proteins were endogenously expressed; italics, cells where proteins were exogenously expressed, surexpressed or mutated

3.1 to 2 h (Lampe, 1994). Hertzberg et al. (2000) compared the exponential decays of both [^{32}P]Pi and [^{35}S]methionine radiolabeled Cx43 in rat leptomeningeal cells and showed that the phosphoprotein turned over with a $t_{1/2}$ of about 1.7 h, whereas the [^{35}S]methionine-radiolabeled mature form of Cx43 had a longer (2.7 h) half-life, suggesting that

phosphorylated Cx43 is a substrate for a rapid proteolytic pathway. When serine residues 263 and 266 of Cx31 were exchanged for amino acids that cannot be phosphorylated, the mutants Cx31Δ263,266 and Cx31Δ266 exhibited half-lives reduced to 56% or 58%, respectively, compared to the wild-type protein (Diestel et al., 2004). Serine residues of the C terminus also represented main phosphorylation sites of mouse Cx45, and when one or both of them at positions 381 and 382 or 384 and 385 were exchanged for other amino acids, the half-life was diminished by 50% compared to wild-type Cx45 (Hertlein et al., 1998). Conversely, mutant Cx45.6 (Ser363 → Ala) expressed in lens primary cultures, whose *in vivo* phosphorylation at Ser363 was prevented, had a longer half-life compared with wild-type Cx45.6 (3 *vs*. 2.5 h; Yin et al., 2000). Laird et al. (1995) observed that, when pulse-chase studies with rat BICR-M1Rk cells were performed in the presence of brefeldin A (BFA), little degradation of the slowest gel migrating Cx43 isoforms (42–43 kDa) occurred, even after 5 h, suggesting that Cx43 trapped within the endoplasmic reticulum (ER) and Golgi compartments was not subjected to degradation. In communication-deficient S180 and L929 cells, which do not phosphorylate Cx43 to the P2 form, Cx43 turns over rapidly ($t_{1/2}$ 1.5–2 h) (Musil et al., 1990).

The rate of membrane protein trafficking is frequently coupled to signaling events. Mitra et al. (2006) recently observed that, in human prostate cancer cells, androgens (testosterone or synthetic androgens) regulated the formation and degradation of gap junctions by rerouting the pool of Cx32, which normally would have been degraded from the early secretory compartment, to the cell surface and enhancing assembly into gap junctions. In cultured bovine aortic endothelial cells, transforming growth factor-β1 treatment caused upregulation of Cx43 synthesis, content and apparent half-life (Larson et al., 1997). An illustration of the possibilities of dynamic regulation of the membrane protein turnover was given in neuromuscular junctions in living adult mice, in which the half-life of the acetylcholine receptors, present at high density, is relatively long (14 days) but considerably shortened (25 times) when neurotransmission is blocked (Akaaboune et al., 1999).

Mutations in the genes that encode the membrane proteins result in a variety of disorders, including their turnover. The Y286A substitution markedly increased the half-life of Cx43 (from 2 to 6 h), suggesting that the increased steady-state levels reflected reduced protein degradation (Thomas et al., 2003). As conserved putative Dab2 binding motifs of the type XPXY are present in the C terminus of Cx43 (P283PGY286) and of at least eight additional mouse and human Cxs (hCx31.9 and its mouse orthologue mCx30.2, h/mCx32, h/mCx37, h/mCx45, h/mCx46, h/mCx47, h/mCx50 and hCx59), Piehl et al. (2007) recently suggested a potential direct interaction of Dab2 with a number of Cxs. In this hypothesis, mutations of critical amino acid residues within and around the putative Dab2 binding site would significantly increase the half-life of Cxs by inhibiting their internalization. When one or both of the serine residues of Cx45 at positions 381 and 382 or 384 and 385 were exchanged for other amino acids, the half-life was diminished by 50% compared to wild-type Cx45 (Hertlein et al., 1998). A mutant human Cx50 associated with cataract (hCx50P88S) accumulates in transfected cells and shows decreased degradation when compared to wild type (Berthoud et al., 2003). More than 130 different mutations in Cx32 have been linked to the human peripheral neuropathy X-linked Charcot-Marie-Tooth disease (CMTX), but the mechanisms remain unclear. VanSlyke et al. (2000) examined the fates of three of these mutants expressed in PC12 cells; one (E208K) remained in the ER, whereas the two others (E186K and R142W) were transported to perinuclear compartments from which one then trafficked to lysosomes (R142W) and the other back to the ER. All of them were unable to assemble into homomeric connexons.

Mutation-caused alterations in the cellular machinery of protein synthesis, folding or degradation are responsible for other severe channelopathies. Deletion of the phenylalanine at position 508 of the CFTR is the most prevalent mutation in cystic fibrosis. This mutation (ΔF508CFTR), which leads to reduced Cl^- conductance, is accompanied by a considerable increase in the turnover rate (the functional half-life of the mutant protein at the plasma membrane is considerably shorter than the one of wild-type CFTR: <4 *vs.* >24 h in K1 cells, Lukacs et al., 1993; 1 *vs.* 3 h in CFBE41o- cells, Swiatecka-Urban et al., 2005; 1 *vs.* 12 h for GFP-CFTR in COS-7 cells, Zhang et al., 2003). Deletion of the PDZ interacting domain reduced the half-life of CFTR in the apical membrane (from approximately 24 to 13 h) but had no effect on the half-life of CFTR in the basolateral membrane, suggesting that the PDZ interacting domain is an apical membrane retention motif (Swiatecka-Urban et al., 2002).

The stability of the wild-type Shaker potassium channels expressed in HEK 293 cells precluded determination of a $t_{1/2}$ value, but the unglycosylated mutant was degraded with a half-life of about 18 h (Khanna et al., 2001).

Functional Consequences for Gap Junctional Communication

Studies involving metabolic labeling and pulse-chase approaches have not formally ruled out the possibility that the observed turnover of Cxs might mainly concern an intracellular pool of proteins, some or all proteins present in gap junction plaques being long-lived enough that they were

not labeled during relatively brief pulse intervals. The advent of protein tags provided undisputable evidence of the dynamic nature of Cxs in cell-surface gap junctions (Jordan et al., 1999; Holm et al., 1999). Time-lapse videomicroscopy allowed observation of the continuous transport of apparently newly synthesized Cx43-GFP to the plasma membrane, the aggregation of the chimeric channel protein into gap junctions at newly formed cell-cell contacts and the removal of Cx43-GFP from the plasma membrane by budding and internalization and usually the formation of distinct endocytic vesicles of different sizes (Jordan et al., 1999). New connexons inserted into nonjunctional areas of the plasma membrane diffuse laterally, joining the periphery of preexisting junctional plaques, whereas older paired connexons are removed from the plaque center. After disruption of the secretory pathway continuum (e.g., by BFA), junctional plaque renewal continues for up to 20 min, presumably due to the arrival of connexons still in transit to the plasma membrane (*for review, see* Martin & Evans, 2004). Direct evidence of the importance of the highly dynamic Cx turnover in living cells and the consequences of interruption by BFA and monensin of intracellular Cx trafficking can be provided by examining in real time the functional consequences of the interruption of transport of newly synthesized Cxs to the plasma membrane by means of fluorescence recovery after photobleaching (FRAP) analysis. In this approach, the junctional permeability for a fluorescent dye is quantified by analyzing the fluorescence emission of a bleached cell connected with dye-loaded neighbor cells (*see* Délèze et al., 2001).

The graphs presented Figure 1 (Bahbouhi, Plaisance, Sarrouilhe & Hervé, unpublished observations) represent typical examples of the evolution with time of emission intensities after photobleaching, measured in cardiac myocytes of newborn rats before and after BFA (20 μM for 2 h, Fig. 1a) or monensin (20 μM for 2 h, Fig. 1b) treatments. Immediately after photobleaching the selected cell, its light emission was markedly reduced, then a rise in fluorescent emission took place with a monoexponential time course, much quicker before (Fig. 1a, b, filled square symbols) than after (empty square symbols) exposure to the drug.

The rate constant k (the inverse of the time constant, *see* Délèze et al., 2001) provides an estimation of the junctional permeability and was found, in control experiments, unmodified when up to four consecutive photobleaches were performed on the same cells (Verrecchia & Hervé, 1997). In control conditions (Tyrode's solution), k was 0.28 ± 0.06 min^{-1} ($n = 19$). In other words, the fluorescence recovery after photobleaching then followed an exponential time course with a mean time constant of approximately 3.6 min.

Both BFA and monensin slowed down the rate of fluorescence recovery, and k was reduced within 2 h to

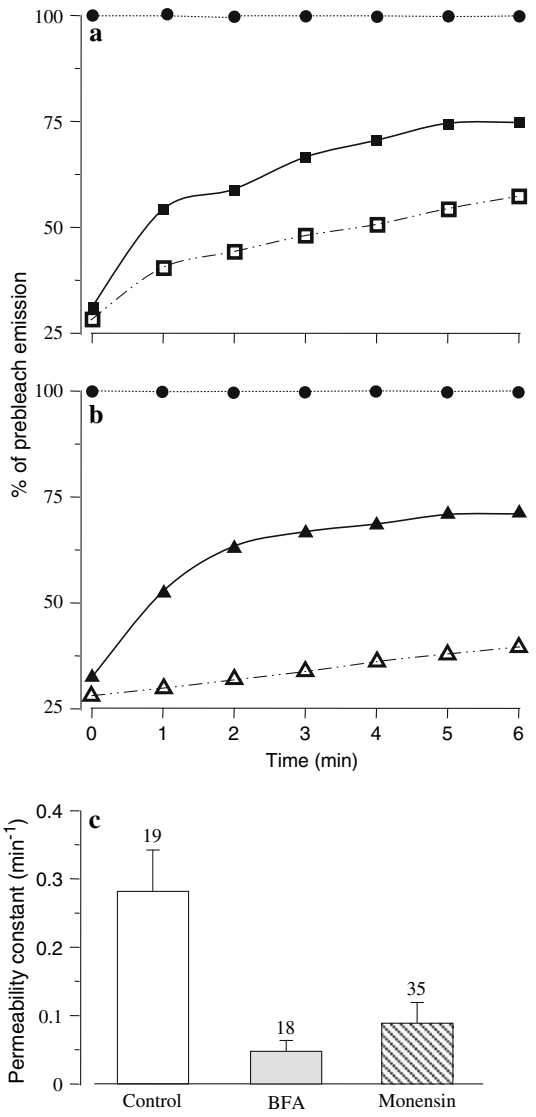

Fig. 1 Golgi-disturbing agents BFA and monensin considerably slowed down cell-to-cell dye diffusion. **a, b** Typical examples of the time courses of the fluorescent emission in bleached cells before (*filled squares* or *triangles* and *continuous line*) and after (*empty squares* or *triangles* and *discontinuous lines*) exposure to BFA (20 μM, *top*) or monensin (20 μM, *middle*) for 2 h. The fluorescent emission of an isolated unbleached cell remained constant (*filled circles* and *dotted lines*). The light emission is represented in percent of the prebleach emission *vs.* time after photobleaching. **c** Relative permeability constant (k) of cell-to-cell dye transfer measured in control conditions (*left column*) and after exposure of cells to BFA (20 μM, *middle column*) or monensin (20 μM, *right column*) for 5 h, with (n) numbers on top

0.14 ± 0.03 ($n = 8$) and 0.11 ± 0.01 ($n = 10$) min^{-1}, respectively, reflecting a reduction in the number of permeable junctional channels. When incubation time with the drugs was prolonged to 5 h, higher inhibition levels were obtained and k was lowered to 0.05 ± 0.02 min^{-1} ($n = 18$) with BFA and 0.09 ± 0.03 min^{-1} ($n = 35$) with monensin (Fig. 1c). By comparison to untreated cells, the difference

was statistically significant with both BFA ($p = 0.013$) and monensin ($p = 0.018$).

In the same cell type, the hindrance of the protein secretory pathway by BFA or monensin dramatically reduced the surface Cx43 staining whereas cytoplasmic staining was increased (Puranam et al., 1993), and the same observation was reported in BWEM cells, which were originally derived from embryonic rat cardiac myocytes (Laing et al., 1998). The data provided by these approaches confirm both that rapid synthesis and trafficking of Cx43 to plasma membrane are required to maintain GJIC and that the conventional Golgi pathway is used in these cells by Cx43 for its translocation to the plasma membrane. They also show that, besides the different mechanisms modulating the gating of junctional channels, the degree of junctional coupling can be directly regulated by the rapid Cx turnover kinetics.

The importance of this phenomenon varies with the type of Cxs forming the channels. A cell-to-cell dye diffusion to two or more of their neighbors was observed after intracellular injection of a fluorescent dye in about 50% of transfected HeLa cells expressing Cx26, Cx32 or Cx43 (respectively, 44%, 51% and 49 %). BFA exposure (17.8 μM) had no effect on the intercellular coupling between Cx26-HeLa cells, even after prolonged treatment (6 h), whereas it markedly reduced the dye transfer between Cx32- and Cx43-HeLa cells (*see* Martin & Evans, 2004), SKHep1 cells (Thomas et al., 2003) and Cx31-HeLa cells (He et al., 2005). BFA (0.3–10 μM) was also seen to gradually decrease GJIC in both Syrian hamster embryo and Wistar rat embryo cells (Cruciani et al., 2003). BFA treatment (7.1 μM, 3 h) reduced by about 45% the intercellular diffusion of Lucifer yellow between rat heart-derived BWEM cells (Laing et al., 1997). Exposure of neonatal rat cardiomyocytes to monensin 10 μM resulted in a 26% decrease of the cell-to-cell diffusion of Lucifer yellow after 4–7 h, whereas longer treatments (7–15 h) caused a stronger reduction (55%; Puranam et al., 1993). Both functional approaches and pulse-chase assays indicate that the rate of Cx replacement is critical for the steady-state level of intercellular junctional communication.

Over the life of an organism, cells face many changes in their environment that result in rearrangements of their membrane; the rate of membrane protein trafficking is critical for the steady-state level of surface expression and is tightly coupled to signaling events. For a given protein (membrane receptor, channel, etc.), evidence is accumulating that the rate of trafficking may change according to the physiological state, such as during cellular aging. In human umbilical vein endothelial cells, e.g., a decline in the level of Cx43 mRNA at the same time as a decrease in the strength of GJIC were observed with age (Xie & Hue, 1994).

Conclusion

Cx proteins, like many other integral plasma membrane proteins (including proteins of other channels, of other intercellular junctions as well as of various membrane receptors), exhibit relatively rapid turnover kinetics, with short half-lives of a few hours. Such a life span has been well documented in both cultured cells and three-dimensional milieus presented by native tissue environments. These membrane proteins have signaling sequences that direct the rate and regulation of their synthesis and release from the ER to govern their insertion into the plasma membrane, where they remain for a quantifiable time period prior to degradation and new protein synthesis. The reasons for this preprogram remain elusive but may include the ability to constantly answer to physiological requirements to either up- or downregulate their activity. One example is given by the myometrium, where a change in the balance of steroid hormones would cause a dramatic increase in total gap junctions just prior to labor onset, followed by rapid clearing.

The extent of intercellular communication depends on the number of junctional channels, their individual permeability and their open probability. In accordance with pulse-chase data, functional approaches show that junctional components are rapidly removed from the plasma membrane even when the translocation of Cxs from intracellular sites to the plasma membrane is interrupted. This transfer is essential to compensate for channel withdrawal from the plasma membrane to maintain and modulate coupling between cells. Protein turnover and degradation appear to represent significant mechanisms for the regulation of GJIC, particularly in the mid- and long term. Some of the observed inhibitory effects of the interruption of protein trafficking (by BFA or monensin) might, however, also be due to a modification of Cx protein partner trafficking. Several reports have indeed suggested that Cxs interact with different proteins to mediate communication (*see*, e.g., Hervé et al., 2007), and such protein partners might be important for the cell to fine-tune the synthesis and degradation of junctional channels. The constant renewal of junctional channels thus provides a means for cells to rapidly modulate gap junctional crosstalk.

Little is known about how protein turnover is controlled; protein synthesis and destruction could be separately regulated. Differences in half-lives may be a direct result of the primary amino acid sequence or may be further modulated by sequence-specific posttranslational modifications.

A wide range of inherited diseases, termed "channelopathies," are caused by protein mutations responsible for defects in the biosynthetic processing and trafficking of different channel-forming proteins (e.g., CFTR for cystic

fibrosis, voltage-gated calcium or sodium channels for calcium channelopathies or sodium channelopathies, etc.). Intracellular mislocations of different Cxs are also associated with different genetic diseases, e.g., CMTX, a form of hereditary motor and sensory neuropathy, caused by mutations in the gene for Cx32. According to Deschenes et al. (1997), CMTX mutations would have a predominant effect on Cx32 trafficking, resulting in its potentially toxic cytoplasmic accumulation (e.g., in the Golgi apparatus, ER or lysosomes). In a human cataract associated with a mutation of Cx50, a significant proportion of the non-functional Cx50 mutant was observed in cytoplasmic accumulations (Berthoud et al., 2003). It was also observed that, in rat neonatal cardiac myocytes in culture infected with *Trypanosoma cruzi* (Chagas disease), although total Cx43 was normal, little was recognizable at appositional membranes; the resulting decreased coupling between cells was presumably involved in the observed cardiac conduction disturbances and arrhythmias (de Carvalho et al., 1994). The increased degradation of Cx43 and the resulting impairment of GJIC activity between vascular endothelial cells appear to be involved in the breakdown of the blood-retinal barrier associated with diabetic retinopathy (*see* Fernandes et al., 2004).

In conclusion, the intercellular coupling strength appears dynamically modulated, the channel-forming proteins (Cxs) being constantly replaced with half-lives comparable to those of several other plasma membrane channels or of transmembrane protein constituents of other intercellular junctions.

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